SECTION 1

INTRODUCTION

1.1 This manual describes chronic toxicity tests for use in the National Pollutant Discharge Elimination System (NPDES) Permits Program to identify effluents and receiving waters containing toxic materials in chronically toxic concentrations. The test methods are also suitable for determining the toxicity of specific compounds contained in discharges. The tests may be conducted in a central laboratory or on-site, by the regulatory agency or the permittee.

1.2 The data are used for NPDES permits development and to determine compliance with permit toxicity limits. Data can also be used to predict potential acute and chronic toxicity in the receiving water, based on hypothesis testing or point estimate techniques (see Section 9, Chronic Toxicity Test Endpoints And Data Analysis) and appropriate dilution, application, and persistence factors. The tests are performed as a part of self-monitoring permit requirements, compliance biomonitoring inspections, toxics sampling inspections, and special investigations. Data from chronic toxicity tests performed as part of permit requirements are evaluated during compliance evaluation inspections and performance audit inspections.

1.3 Modifications of these tests are also used in toxicity reduction evaluations and toxicity identification evaluations to identify the toxic components of an effluent, to aid in the development and implementation of toxicity reduction plans, and to compare and control the effectiveness of various treatment technologies for a given type of industry, irrespective of the receiving water (USEPA, 1988c; USEPA, 1989b; USEPA, 1989c; USEPA, 1989d; USEPA, 1989e; USEPA, 1991a; USEPA, 1991b; USEPA, 1992).

1.4 This methods manual serves as a companion to the acute toxicity test methods for freshwater and marine organisms (USEPA, 1993a), the short-term chronic toxicity test methods for freshwater organisms (USEPA, 1993b), the short-term chronic toxicity test methods for east coast organisms (USEPA, 1994), and the manual for evaluation of laboratories performing aquatic toxicity tests (1991c).

1.5 Guidance for the implementation of toxicity tests in the NPDES program is provided in the Technical Support Document for Water Quality-Based Toxics Control (USEPA, l991a).

1.6 These marine and estuarine short-term toxicity tests are similar to those developed for the freshwater organisms and east coast marine organisms to evaluate the toxicity of effluents discharged to estuarine and coastal marine waters under the NPDES permit program. Methods are presented in this manual for ten species from six phylogenetic groups. The red abalone larval development test method, the giant kelp germination and germ-tube length test method, the mysid survival and growth test method and the topsmelt survival and growth test method were developed and extensively field tested by University of California, Santa Cruz through the California State Water Resources Control Board's Marine Bioassay Project. The purple urchin and sand dollar fertilization test method was developed by U.S. Environmental Research Laboratory-Newport, Oregon. The purple urchin and sand dollar development test method was developed by the Southern California Coastal Water Research Project. The Pacific oyster and mussel survival and larval development test method was modified from ASTM 1989 by the Washington Department of Ecology and the USEPA. The methods vary in duration from 40 minutes to seven days.

1.7 The ten species for which toxicity test methods provided are: the topsmelt, *Atherinops affinis*, the red abalone, *Haliotis rufescens*; the Pacific oyster, *Crassostrea gigas*, mussel *Mytilus spp.*; the mysid, *Holmesimysis costata*; the sea urchin, *Strongylocentrotus purpuratus*, the sand dollar, *Dendraster excentricus*; and the giant kelp, *Macroystis pyrifera*.

1.7.1 Many of the tests included in this document are based on the following:

1. "Marine Bioassay Project Seventh Reports (Reports 1-7)" by Brian S. Anderson, John W. Hunt, and Hilary R. McNulty, University of California, Santa Cruz; Mark D. Stephenson, California Department of Fish and Game; and Francis H. Palmer, Debra L. Denton, and Matthew Reeve, State Water Resources Control Board.

2. "Procedures Manual for Conducting Toxicity Tests Developed by the Marine Bioassay Project by Brian S. Anderson, John W. Hunt, Shiela L. Turpen, A.R. Coulon, University of California, Santa Cruz; Mike Martin, California of Department of Fish and Game; Debra L. Denton and Frank H. Palmer, State Water Resources Control Board, 90-10WQ, 112 pp.

3. "Standard Practice for Conducting Static Acute Toxicity Tests with Larvae of Four Species of Bivalve Molluscs. ASTM 1989.

1.7.2 Three of the methods incorporate the chronic endpoints of growth or development (or both) in addition to lethality. The sea urchin sperm cell test uses fertilization as an endpoint and has the advantage of an extremely short exposure period (40 minutes).

1.8 The validity of similar marine/estuarine methods in predicting adverse ecological impacts of toxic discharges was demonstrated in field studies (USEPA, 1986d).

1.9 The use of any marine or estuarine test species or test conditions other than those described in the methods summary tables in this manual or in the east coast marine manual (USEPA/600/4-91/003) shall be subject to application and approval of alternate test procedures under 40 CFR 136.4 and 40 CFR 136.5.

1.10 These methods are restricted to use by or under the supervision of analysts experienced in the use or conduct of aquatic toxicity testing and the interpretation of data from aquatic toxicity testing. Each analyst must demonstrate the ability to generate acceptable test results with these methods using the procedures described in this methods manual.

1.11 The manual was prepared in the established NERL-Cincinnati format (USEPA, 1983).

SECTION 2

SHORT-TERM METHODS FOR ESTIMATING CHRONIC TOXICITY

2.1 INTRODUCTION

2.1.1 The objective of aquatic toxicity tests with effluents or pure compounds is to estimate the "safe" or "no-effect" concentration of these substances, which is defined as the concentration which will permit normal propagation of fish and other aquatic life in the receiving waters. The endpoints that have been considered in tests to determine the adverse effects of toxicants include death and survival, decreased reproduction and growth, locomotor activity, gill ventilation rate, heart rate, blood chemistry, histopathology, enzyme activity, olfactory function, and terata. Since it is not feasible to detect and/or measure all of these (and other possible) effects of toxic substances on a routine basis, observations in toxicity tests generally have been limited to only a few effects, such as mortality, growth, and reproduction.

2.1.2 Acute lethality is an obvious and easily observed effect which accounts for its wide use in the early period of evaluation of the toxicity of pure compounds and complex effluents. The results of these tests were usually expressed as the concentration lethal to 50% of the test organisms (LC50) over relatively short exposure periods (one-to-four days).

2.1.3 As exposure periods of acute tests were lengthened, the LC50 and lethal threshold concentration were observed to decline for many compounds. By lengthening the tests to include one or more complete life cycles and observing the more subtle effects of the toxicants, such as a reduction in growth and reproduction, more accurate, direct, estimates of the threshold or safe concentration of the toxicant could be obtained. However, laboratory life cycle tests may not accurately estimate the "safe" concentration of toxicants because they are conducted with a limited number of species under highly controlled, steady state conditions, and the results do not include the effects of the stresses to which the organisms would ordinarily be exposed in the natural environment.

2.1.4 An early published account of a full life cycle, fish toxicity test was that of Mount and Stephan (1967). In this study, fathead minnows, *Pimephales promelas*, were exposed to a graded series of pesticide concentrations throughout their life cycle, and the effects of the toxicant on survival, growth, and

reproduction were measured and evaluated. This work was soon followed by full life cycle tests using other toxicants and fish species.

2.1.5 McKim (1977) evaluated the data from 56 full life cycle tests, 32 of which used the fathead minnow, *Pimephales promelas*, and concluded that the embryo-larval and early juvenile life stages were the most sensitive stages. He proposed the use of partial life cycle toxicity tests with the early life stages (ELS) of fish to establish water quality criteria.

2.1.6 Macek and Sleight (1977) found that exposure of critical life stages of fish to toxicants provides estimates of chronically safe concentrations remarkably similar to those derived from full life cycle toxicity tests. They reported that "for a great majority of toxicants, the concentration which will not be acutely toxic to the most sensitive life stages is the chronically safe concentration for fish, and that the most sensitive life stages are the embryos and fry." Critical life stage exposure was considered to be exposure of the embryos during most, preferably all, of the embryogenic (incubation) period, and exposure of the fry for 30 days post-hatch for warm water fish with embryogenic periods ranging from one-to-fourteen days, and for 60 days post-hatch for fish with longer embryogenic periods. They concluded that in the majority of cases, the maximum acceptable toxicant concentration (MATC) could be estimated from the results of exposure of the embryos during incubation, and the larvae for 30 days post-hatch.

2.1.7 Because of the high cost of full life-cycle fish toxicity tests and the emerging consensus that the ELS test data usually would be adequate for estimating chronically safe concentrations, there was a rapid shift by aquatic toxicologists to 30- to 90-day ELS toxicity tests for estimating chronically safe concentrations in the late 1970s. In 1980, USEPA adopted the policy that ELS test data could be used in establishing water quality criteria if data from full life-cycle tests were not available (USEPA, 1980a).

2.1.8 Published reports of the results of ELS tests indicate that the relative sensitivity of growth and survival as endpoints may be species dependent, toxicant dependent, or both. Ward and Parrish (1980) examined the literature on ELS tests that used embryos and juveniles of the sheepshead minnow, *Cyprinodon variegatus*, and found that growth was not a statistically sensitive indicator of toxicity in 16 of 18 tests. They suggested that the ELS tests be shortened to 14 days posthatch and that growth be eliminated as an indicator of toxic effects.

2.1.9 In a review of the literature on 173 fish full life-cycle and ELS tests performed to determine the chronically safe concentrations of a wide variety of toxicants, such as metals, pesticides, organics, inorganics, detergents, and complex effluents, Woltering (1984) found that at the lowest effect concentration, significant reductions were observed in fry survival in 57%, fry growth in 36%, and egg hatchability in 19% of the tests. He also found that fry survival and growth were very often equally sensitive, and concluded that the growth response could be deleted from routine application of the ELS tests. The net result would be a significant reduction in the duration and cost of screening tests with no appreciable impact on estimating MATCs for chemical hazard assessments. Benoit et al. (1982), however, found larval growth to be the most significant measure of effect and survival to be equally or less sensitive than growth in early life-stage tests with four organic chemicals.

2.1.10 Efforts to further reduce the length of partial life-cycle toxicity tests for fish without compromising their predictive value have resulted in the development of an eight-day, embryo-larval survival and teratogenicity test for fish and other aquatic vertebrates (USEPA, 1981; Birge et al., 1985), and a seven-day larval survival and growth test (Norberg and Mount, 1985).

2.1.11 The similarity of estimates of chronically safe concentrations of toxicants derived from short-term, embryo-larval survival and teratogenicity tests to those derived from full life-cycle tests has been demonstrated by Birge et al. (1981), Birge and Cassidy (1983), and Birge et al. (1985).

2.1.12 Use of a seven-day, fathead minnow, *Pimephales promelas*, larval survival and growth test was first proposed by Norberg and Mount at the 1983 annual meeting of the Society for Environmental Toxicology and Chemistry (Norberg and Mount, 1983). This test was subsequently used by Mount and associates in field demonstrations at Lima, Ohio (USEPA, 1984), and at many other locations (USEPA, 1985c, USEPA, 1985d; USEPA, 1985e; USEPA, 1986a; USEPA, 1986b; USEPA, 1986c; USEPA, 1986d). Growth was frequently found to be more sensitive than survival in determining the effects of complex effluents.

2.1.13 Norberg and Mount (1985) performed three single toxicant fathead minnow larval growth tests with zinc, copper, and DURSBAN®, using dilution water from Lake Superior. The results

were comparable to, and had confidence intervals that overlapped with, chronic values reported in the literature for both ELS and full life-cycle tests.

2.1.14 USEPA (1987b) and USEPA (1987c) adapted the fathead minnow larval growth and survival test for use with the sheepshead minnow and the inland silverside, respectively. When daily renewal 7-day sheepshead minnow larval growth and survival tests and 28-day ELS tests were performed with industrial and municipal effluents, growth was more sensitive than survival in seven out of 12 larval growth and survival tests, equally sensitive in four tests, and less sensitive in only one test. In four cases, the ELS test may have been three to 10 times more sensitive to effluents than the larval growth and survival test. In tests using copper, the No Observable Effect Concentrations (NOECs) were the same for both types of test, and growth was the most sensitive endpoint for both. In a four laboratory comparison, six of seven tests produced identical NOECs for survival and growth (USEPA, 1987a). Data indicate that the inland silverside is at least equally sensitive or more sensitive to effluents and single compounds than the sheepshead minnow, and can be tested over a wider salinity range, 5-30‰ (USEPA, 1987a).

2.1.15 Lussier et al. (1985) and USEPA (1987e) determined that survival and growth are often as sensitive as reproduction in 28-day life-cycle tests with the mysid, *Mysidopsis bahia*.

2.1.16 Nacci and Jackim (1985) and USEPA (1987g) compared the results from the sea urchin fertilization test, using organic compounds, with results from acute toxicity tests using the freshwater organisms, fathead minnows, *Pimphales promelas*, and *Daphnia magna*. The test was also compared to acute toxicity tests using Atlantic silverside, *Menidia menidia*, and the mysid, *Mysidopsis bahia*, and five metals. For six of the eight organic compounds, the results of the fertilization test and the acute toxicity test correlated well ($r^2 = 0.85$). However, the results of the fertilization test with the five metals did not correlate well with the results from the acute tests.

2.1.17 USEPA (1987f) evaluated two industrial effluents containing heavy metals, five industrial effluents containing organic chemicals (including dyes and pesticides), and 15 domestic wastewaters using the two-day red macroalga, *Champia parvula*, sexual reproduction test. Nine single compounds were used to compare the effects on sexual reproduction using a

two-week exposure and a two-day exposure. For six of the nine compounds tested, the chronic values were the same for both tests.

2.1.18 The use of short-term toxicity tests in the NPDES Program is especially attractive because they provide a more direct estimate of the safe concentrations of effluents in receiving waters than was provided by acute toxicity tests, at an only slightly increased level of effort, compared to the fish full life-cycle chronic and 28-day ELS tests and the 28-day mysid life-cycle test.

2.2 **TYPES OF TESTS**

2.2.1 The selection of the test type will depend on the NPDES permit requirements, the objectives of the test, the available resources, the requirements of the test organisms, and effluent characteristics such as fluctuations in effluent toxicity.

2.2.2 Effluent chronic toxicity is generally measured using a multi-concentration, or definitive test, consisting of a control and a minimum of five effluent concentrations. The tests are designed to provide dose-response information, expressed as the percent effluent concentration that affects the survival, fertilization, growth, and/or development within the prescribed period of time (40 minutes to seven days). The results of the tests are expressed in terms of either the highest concentration that has no statistically significant observed effect on those responses when compared to the controls or the estimated concentration that causes a specified percent reduction in responses versus the controls.

2.2.3 Use of pass/fail tests consisting of a single effluent concentration (e.g., the receiving water concentration or RWC) and a control **is not recommended**. If the NPDES permit has a whole effluent toxicity limit for acute toxicity at the RWC, it is prudent to use that permit limit as the midpoint of a series of five effluent concentrations. This will ensure that there is sufficient information on the dose-response relationship. For example, if the RWC is >25% then, the effluent concentrations utilized in a test may be: (1) 100% effluent, (2) (RWC + 100)/2, (3) RWC, (4) RWC/2, and (5) RWC/4. More specifically, if the RWC = 50%, the effluent concentrations used in the toxicity test would be 100%, 75%, 50%, 25%, and 12.5%. If the RWC is <25% effluent the concentrations may be: (1) 4 times the RWC, (2) 2 times the RWC, (3) RWC, (4) RWC/2, and (5) RWC/4.

2.2.4 Receiving (ambient) water toxicity tests commonly employ two treatments, a control and the undiluted receiving water, but may also consist of a series of receiving water dilutions.

2.2.5 A negative result from a chronic toxicity test does not preclude the presence of toxicity. Also, because of the potential temporal variability in the toxicity of effluents, a negative test result with a particular sample does not preclude the possibility that samples collected at some other time might exhibit chronic toxicity.

2.2.6 The frequency with which chronic toxicity tests are conducted under a given NPDES permit is determined by the regulatory agency on the basis of factors such as the variability and degree of toxicity of the waste, production schedules, and process changes.

2.2.7 Tests recommended for use in this methods manual may be static non-renewal or static renewal. Individual methods specify which type of test is to be conducted.

2.3 **STATIC TESTS**

2.3.1 Static non-renewal tests - The test organisms are exposed to the same test solution for the duration of the test.

2.3.2 Static-renewal tests - The test organisms are exposed to a fresh solution of the same concentration of sample every 24 h or other prescribed interval, either by transferring the test organisms from one test chamber to another, or by replacing all or a portion of solution in the test chambers.

2.4 **ADVANTAGES AND DISADVANTAGES OF TOXICITY TEST TYPES**

2.4.1 STATIC NON-RENEWAL, SHORT-TERM TOXICITY TESTS:

Advantages:

1. Simple and inexpensive.
2. More cost effective in determining compliance with permit conditions.
3. Limited resources (space, manpower, equipment) required; would permit staff to perform more tests in the same amount of time.
4. Smaller volume of effluent required than for static renewal or flow-through tests.

Disadvantages:

1. Dissolved oxygen (DO) depletion may result from high chemical oxygen demand (COD), biological oxygen demand (BOD), or metabolic wastes.
2. Possible loss of toxicants through volatilization and/or adsorption to the exposure vessels.
3. Generally less sensitive than renewal because the toxic substances may degrade or be adsorbed, thereby reducing the apparent toxicity. Also, there is less chance of detecting slugs of toxic wastes, or other temporal variations in waste properties.

2.4.2 STATIC RENEWAL, SHORT-TERM TOXICITY TESTS:

Advantages:

1. Reduced possibility of DO depletion from high COD and/or BOD, or ill effects from metabolic wastes from organisms in the test solutions.
2. Reduced possibility of loss of toxicants through volatilization and/or adsorption to the exposure vessels.
3. Test organisms that rapidly deplete energy reserves are fed when the test solutions are renewed, and are maintained in a healthier state.

Disadvantages:

1. Require greater volume of effluent than non-renewal tests.
2. Generally less chance of temporal variations in waste properties.

SECTION 3

HEALTH AND SAFETY

3.1 GENERAL PRECAUTIONS

3.1.1 Each laboratory should develop and maintain an effective health and safety program, requiring an ongoing commitment by the laboratory management and includes: (1) a safety officer with the responsibility and authority to develop and maintain a safety program; (2) the preparation of a formal, written, health and safety plan, which is provided to the laboratory staff; (3) an ongoing training program on laboratory safety; and (4) regularly scheduled, documented, safety inspections.

3.1.2 Collection and use of effluents in toxicity tests may involve significant risks to personal safety and health. Personnel collecting effluent samples and conducting toxicity tests should take all safety precautions necessary for the prevention of bodily injury and illness which might result from ingestion or invasion of infectious agents, inhalation or absorption of corrosive or toxic substances through skin contact, and asphyxiation due to a lack of oxygen or the presence of noxious gases.

3.1.3 Prior to sample collection and laboratory work, personnel should determine that all necessary safety equipment and materials have been obtained and are in good condition.

3.1.4 Guidelines for the handling and disposal of hazardous materials must be strictly followed.

3.2 SAFETY EQUIPMENT

3.2.1 PERSONAL SAFETY GEAR

3.2.1.1 Personnel must use safety equipment, as required, such as rubber aprons, laboratory coats, respirators, gloves, safety glasses, hard hats, and safety shoes. Plastic netting on glass beakers, flasks and other glassware minimizes breakage and subsequent shattering of the glass.

3.2.2 LABORATORY SAFETY EQUIPMENT

3.2.2.1 Each laboratory (including mobile laboratories) should be provided with safety equipment such as first aid kits, fire extinguishers, fire blankets, emergency showers, chemical spill clean-up kits, and eye fountains.

3.2.2.2 Mobile laboratories should be equipped with a telephone to enable personnel to summon help in case of emergency.

3.3 **GENERAL LABORATORY AND FIELD OPERATIONS**

3.3.1 Work with effluents should be performed in compliance with accepted rules pertaining to the handling of hazardous materials (see safety manuals listed in Section 3, Health and Safety, Subsection 3.5). It is recommended that personnel collecting samples and performing toxicity tests should not work alone.

3.3.2 Because the chemical composition of effluents is usually only poorly known, they should be considered as potential health hazards, and exposure to them should be minimized. Fume and canopy hoods over the toxicity test areas must be used whenever possible.

3.3.3 It is advisable to cleanse exposed parts of the body immediately after collecting effluent samples.

3.3.4 All containers should be adequately labeled to indicate their contents.

3.3.5 Staff should be familiar with safety guidelines on Material Safety Data Sheets for reagents and other chemicals purchased from suppliers. Incompatible materials should not be stored together. Good housekeeping contributes to safety and reliable results.

3.3.6 Strong acids and volatile organic solvents employed in glassware cleaning must be used in a fume hood or under an exhaust canopy over the work area.

3.3.7 Electrical equipment or extension cords not bearing the approval of Underwriter Laboratories must not be used. Ground-fault interrupters must be installed in all "wet" laboratories where electrical equipment is used.

3.3.8 Mobile laboratories should be properly grounded to protect against electrical shock.

3.4 **DISEASE PREVENTION**

3.4.1 Personnel handling samples which are known or suspected to contain human wastes should be immunized against tetanus, typhoid fever, polio, and hepatitis B.

3.5 **SAFETY MANUALS**

3.5.1 For further guidance on safe practices when collecting effluent samples and conducting toxicity tests, check with the permittee and consult general safety manuals, including USEPA (1986e), and Walters and Jameson (1984).

3.6 **WASTE DISPOSAL**

3.6.1 Wastes generated during toxicity testing must be properly handled and disposed of in an appropriate manner. Each testing facility will have its own waste disposal requirements based on local, state and Federal rules and regulations. It is extremely important that these rules and regulations be known, understood, and complied with by all persons responsible for, or otherwise involved in, performing toxicity testing activities. Local fire officials should be notified of any potentially hazardous conditions.